Targeted Delivery of an Antibody–Mutant Human Endostatin Fusion Protein Results in Enhanced Antitumor Efficacy

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Abstract

The antiangiogenic protein endostatin showed considerable preclinical antitumor activity, but limited efficacy in phase I/II trials. Prior studies using an anti-HER2 antibody–murine endostatin fusion showed enhanced antitumor activity compared to anti-HER2 antibody or endostatin given alone, or in combination. We have generated two anti-HER2 human endostatin fusion proteins by fusing either wild-type or a mutant human endostatin (huEndo-P125A) to the 3' end of a humanized anti-HER2 IgG3 antibody. Antitumor efficacy was examined in murine and human breast tumor models. HuEndo-P125A antibody fusion protein (αHER2-huEndo-P125A) inhibited VEGF and bFGF induced endothelial cell proliferation, and tube formation in vitro, more efficiently than endostatin alone, wild-type endostatin fusion protein (αHER2-huEndo), or parental anti-HER2 antibody (αHER2 IgG3). Wild-type and mutant human endostatin was rapidly cleared from serum in mice (T½ 2 = 2.0–2.1 hours), whereas αHER2-huEndo fusion proteins had a significantly prolonged half-life (T½ 2 = 40.7–57.5 hours). Treatment of SK-BR-3 breast cancer xenografts with anti-HER2 IgG3-huEndo-P125A fusion resulted in greater inhibition of tumor growth and improved survival, compared to treatment with either αHER2 IgG3 (P = 0.025), human endostatin (P = 0.034), or anti-HER2 IgG3-huEndo (P = 0.016). αHER2-huEndo-P125A specifically inhibited tumors expressing HER2 in mice simultaneously implanted with murine mammary tumor EMT6 cells and with EMT6 engineered to express HER2 antigen (EMT6-HER2). Targeting of endostatin using antibody fusion proteins could improve antitumor activity of either anti-HER2 antibody and/or endostatin and provides a versatile approach that could be applied to other tumor targets with alternative antibody specificities.

Mol Cancer Ther; 10(4); 603–14. ©2011 AACR.

Introduction

Endostatin, an antiangiogenic fragment of collagen XVIII, is a global inhibitor of endothelial cell proliferation in vitro and angiogenesis in vivo (1). The mechanism of action of endostatin is not fully understood. Systemic therapy with murine endostatin (mEndo) inhibited the growth of Lewis lung carcinoma, fibrosarcomas (T241), melanomas (B16F10), hemangioepithelioma (EOMA; refs. 1 and 2), and human renal cell cancer xenografts (3). Human and mouse collagen XVIII chains show a high degree of homology (4). Human endostatin (huEndo) inhibited the growth of several different tumors in vivo, such as human glioblastoma (U-87MG), C6 rat glioma, or rat gliosarcoma (BT4Cn; refs. 5–7). Repeated treatment with endostatin led to permanent eradication of tumor in several rodent models (1, 2, 8).

Although initial trials proved that endostatin is safe when delivered in a variety of dose schedules, they did not show comparable antitumor activity to that seen in murine models. In human phase I trials, huEndo administration at variable dose schedules was feasible and safe but, little or no consistent antitumor activity was shown (9–13). In a phase II study in 42 patients with advanced pancreatic neuroendocrine tumors or carcinoid tumors treated with huEndo administered as a subcutaneous injection, huEndo showed minimal toxicity (13), but no patient achieved a partial response.

Several explanations have been advanced for the failure to see antitumor activity in the human setting. It has been suggested that initial preparations of endostatin had suboptimal biologic activity and a modified formulations (e.g., EndoStar) may be more potent and have been approved for use in China (14–16). Endostatin may be
more potent when delivered as a dimer or trimer than when delivered in monomeric form (17, 18). Finally, a mutant form of endostatin (P125A) has been reported to have enhanced antiangiogenic activity (19–21). Endostatin has also been reported to show a bimodal response curve such that optimal concentrations may not have been achieved in phase I/II studies (22).

To improve efficacy of trastuzumab and endostatin, we constructed several endostatin fusion proteins by joining human endostatin to the 3‘ end of humanized anti-HER2 IgG3. We reasoned that delivery of endostatin using an antibody fusion protein would increase endostatin half-life, enhance antiangiogenic activity due to endostatin delivery as a dimer, and selectively deliver endostatin to the site of tumor, thereby improving efficacy. We had previously validated the efficacy of this approach using an anti-HER2 antibody fused to murine endostatin. In this study, we report on the biological activity of an αHER2 IgG3 fused to either a wild type or a mutant form (P125A) of human endostatin with markedly enhanced antiangiogenic activity. We show that this fusion protein is associated with significantly enhanced antiangiogenic and antitumor efficacy in several cancer models.

**Materials and Methods**

**Cell lines, materials, and animals**

Murine mammary tumor EMT6 cells were transduced with a retroviral vector encoding human HER2 as described (23, 24). EMT6, SK-BR-3, Sp2/0, and P3×63Ag8.653 cells were purchased from and characterized by the American Type Culture Collection. EMT6, EMT6-HER2, the human breast cancer cell line SK-BR-3, and transfected Sp2/0 or P3×63Ag8.653 cells were cultured in Iscove’s Modified Dulbecco’s Medium with 5% calf serum. Expression of HER2 was confirmed on EMT6-HER2 and SK-BR-3 cell lines using flow cytometry. Sp2/0 and P3×63Ag8.653 were tested by ELISA for antibody expression. Human recombinant endostatin was purchased from commercial sources (E81154, Sigma). Female BALB/c mice (4–6 weeks) and severe combined immunodeficient (SCID) mice (4–6 weeks, Jackson Laboratory) were used for in vivo tumor growth and xenograft experiments (SK-BR-3) as indicated. All experiments were conducted in compliance with the NIH Guides for the Care and Use of Laboratory Animals and approved by the University of Miami Institutional Animal Care and Use Committee.

**Construction, expression, and characterization of αHER2-huEndo fusion protein**

The huEndo gene was cloned from the human collagen, type XVIII, α1 gene by PCR using primers 5’-CCCCTGCAGATATCACCCACCCCGCATTCCAG-CGG-3’ and 5’-CCCCGATTCTTTGATACCTTGAGG-GAGTCATGAAAC-3’. PCR products were subcloned into pCR-Blunt II-TOPO vector. A single point mutation in human endostatin at position 125 (proline to alanine; huEndo-P125A) has been reported to enhance antiangiogenic activity (19–21). A mutant clone in wild-type human endostatin encoding an alanine residue substituted for proline at position 125 was derived by site-directed mutagenesis using PCR with phosphorylated primer, 5’-GGCTCGGAGCCAAACGGGC-3’. The subcloned huEndo and huEndo-P125A genes were ligated in frame to the carboxyl end of the heavy chain constant domain of human IgG3 in the vector pAT135 (24, 25). The endostatin heavy chain constant region was joined to the C-terminus of the anti-HER2 heavy chain of a recombinant humanized monoclonal antibody 4D5–8 (Genentech) in the expression vector (pSV2-his) containing a HisD gene for eukaryotic selection (23, 26).

The αHER2-huEndo fusion constructs were stably transfected into SP2/0 or P3×63Ag8.653 myeloma cells expressing the anti-HER2 κ light chain by electroporation as described previously (27). The endostatin fusion proteins were purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (yield: 1–5 mg/L of supernatant; Sigma) as described (27). αHER2-huEndo fusion proteins were biosynthetically labeled with [35S]methionine (Amersham Biosciences), immunoprecipitated using IgGsorb suspension, and analyzed by SDS-PAGE as described (27).

P125A-endostatin was cloned and expressed in Pichia pastoris (20). Following methanol based induction of the transgene, P125A-endostatin was purified from culture supernatants using heparin linked ceramic particles (20).

**Flow cytometry**

To detect the binding of αHER2-huEndo fusion proteins to HER2 antigen and human umbilical vein endothelial cells (HUVEC; Clontech Lab) human SK-BR-3 breast cancer cells, murine mammary tumor cells, EMT6 and EMT6-HER2, and HUVECs were incubated at 4°C with 1 μg/mL of endostatin fusion proteins, αHER2 IgG3, or isotype control. Fifteen minutes later, the cells were washed with PBS containing 0.1% BSA and 0.05% NaN3, and the bound fusion proteins were identified with either FITC conjugated antihuman IgG, or the endostatin antibody and streptavidin conjugated (Sigma) at 4°C, washed twice and resuspended in PBS and analyzed by flow cytometry. Background staining was estimated following incubation with the secondary FITC or PE-labeled antibody alone.

Binding to HUVECs was used to determine endostatin domain binding, using a biotinylated antihuman endostatin antibody and streptavidin conjugated PE as indicated earlier.

**Matrigel tube formation assay**

HUVECs were used between passages 3 and 5, and maintained in EGM2-MV medium (Clontech) that contained endothelial basal medium 2 (EBM-2), supplemented with 2% fetal bovine serum, gentamicin, amphotericin

Published OnlineFirst March 10, 2011; DOI: 10.1158/1535-7163.MCT-10-0804
B, hydrocortisone, ascorbic acid, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), human epidermal growth factor, and insulin-like growth factor I as described (28, 29).

The Matrigel tube formation assay was done in 48-well plates, as previously reported (28, 29). Each well of prechilled 48-well cell culture plates was coated with 100 μL of unpolymerized Matrigel (7 mg/mL) and incubated at 37°C for 30–45 minutes. HUVECs were harvested with trypsin, and 4 x 10^4 cells were resuspended in 300 μL of full EC growth medium and treated with endostatin, control antibody or the various αHER2-huEndo fusion proteins at indicated concentrations before plating onto the Matrigel-coated plates. Following 16 hours of incubation at 37°C, EC tube formation was assessed with an inverted photomicroscope, and microphotographs of the center of each well were taken at low power (40×). Tube formation by untreated HUVEC in full EC growth medium was used as a control.

**Pharmacokinetics of αHER2-huEndo fusion proteins**

To monitor the levels of human endostatin fusion proteins in serum, BALB/c mice (n = 3, 4–6 weeks) were injected i.v. with either αHER2-huEndo (50 μg, equimolar to 9.52 μg human endostatin), αHER2-huEndo-P125A (50 μg, equimolar to 9.52 μg human endostatin), huEndo (10 μg), or huEndo-P125A (10 μg). Blood samples were serially obtained at indicated intervals αHER2-huEndo or αHER2-huEndo-P125A. Mice injected with either huEndo or huEndo-P125A was bled within 1 minute to 2 hours after the i.v. injection. A quantitative ELISA for serum endostatin (Quantikine Human ES ELISA kit, R&D Systems) was used to detect both endostatin and endostatin fusion proteins according to the manufacturer’s instructions.

The pharmacokinetic variables were calculated by fitting human endostatin concentration data in serum to a biexponential model with derivative free nonlinear regression analysis (PK Solution, version 2.0.6 Program developed at Summit Research Services. The pharmacokinetic variables, such as serum distribution and elimination, steady-state area under the serum concentration curve (AUC_{0-∞}), and mean residence time, were calculated.

**In vivo tumor growth assays**

To evaluate antitumor activity of αHER2-huEndo fusion proteins in vivo, human SK-BR-3 breast cancer cells (2 x 10^6) were implanted s.c. in the flanks of SCID mice (24). Starting on day 4, mice were injected i.v. with αHER2-huEndo-P125A (42 μg/injection), or PBS as a control every 2 days. On day 12, mice were sacrificed for analysis of vascularity following 4 of the aforementioned treatments. Tumors were excised from the sacrificed mice, frozen in liquid nitrogen and 8 μm frozen sections were prepared. The tumor sections were fixed with methanol for 10 minutes, washed with PBS 3 times, and incubated with blocking solution for 1 hour in a humidified chamber and again washed with PBS 3 times. For immunofluorescent staining, diluted primary antibodies [rat anti-mouse CD31 antibody conjugated with biotin, 1:200 in PBS (BD Biosciences)] were added to each slide. Following incubation at room temperature overnight, sections were incubated with diluted secondary antibodies conjugated with Alexa Fluor 488 (1:500) with PBS, and then with diluted DAPI (1:5000) in a humidified chamber and mounted with Gel mounting media (Biomeda Corp.). Alexa Fluor 488 was obtained from In vitrino, and DAPI from Molecular Probes. The stained images from 5 low power fields were analyzed with a Zeiss microscope. Digital tumor images from each treatment were measured for blood vessel area (pixel^2), averaged to measure blood vessel density per tumor, and vessel number and density analyzed using NIH ImageJ software.

**Statistical analysis**

Statistical analysis was carried out with Graphpad Prism 4 (GraphPad Software, Inc.). HUVEC proliferation and antitumor efficacy in human breast cancer SK-BR-3 xenografts were analyzed by 2-way repeated measures (RM) analyses of variance (ANOVA), followed by
Bonferroni posttest. Tumor growth in the murine syngeneic tumor model was compared using Student’s t test (unpaired, 2-tailed) for each treatment group, and was analyzed among 3 different treatments by 1-way ANOVA, followed by Bonferroni’s multiple comparison test. Graphs were expressed as the mean values with 95% confidence interval (CI). Differences were considered statistically significant at \( P < 0.05 \).

Results

Construction and purification of \( \alpha \)HER2-huEndo fusion proteins

The cloned huEndo and huEndo-P125A genes were genetically fused to the 3’ end of a humanized anti-HER2 IgG3 antibody heavy chain gene. The anti-HER2 IgG3-huEndo fusion protein heavy chain constructs were stably transfected into myeloma cells expressing the anti-HER2 kappa light chain in order to assemble the entire anti-HER2 IgG3-huEndo fusion proteins, anti-HER2 IgG3-huEndo (\( \alpha \)HER2-huEndo) and anti-HER2 IgG3-huEndo-P125A (\( \alpha \)HER2-huEndo-P125A; Fig. 1A). The resulting \( \alpha \)HER2-huEndo and \( \alpha \)HER2-huEndo-P125A fusion proteins were biosynthetically labeled with \[^{35}\text{S} \]methionine and analyzed by SDS-PAGE. \( \alpha \)HER2-huEndo fusion proteins of the expected molecular weight were secreted as the fully assembled \( \text{H}_{2}\text{L}_{2} \) form (Fig. 1B). The secreted \[^{35}\text{S} \]methionine-labeled proteins had a molecular weight of 220 kDa under nonreducing conditions, the size expected for a complete antibody (170 kDa) with 2 molecules of endostatin (25 kDa each) attached (Fig. 1B). Following reduction, heavy and light chains of the expected molecular weight were observed (85 kDa and 25 kDa, respectively; Fig. 1B). The secreted endostatin fusion proteins were then purified from culture supernatants using a protein A column.

Binding to HER2 target antigen and to HUVECs

Human HER2+ SK-BR-3 cells, and murine EMT6 and EMT6-HER2 were used to test whether the endostatin fusion proteins could recognize the HER2 antigen (Fig. 1C). Using anti-human IgG antibody as a detection antibody, \( \alpha \)HER2 IgG3, \( \alpha \)HER2-huEndo, and \( \alpha \)HER2-huEndo-P125A, bound to HER2+ SKBR3 and EMT6-HER2 cells (Fig. 1C-I and II, respectively), whereas the isotype control antibody (anti-dansyl IgG3) did not bind.

\( \alpha \)HER2 IgG3, \( \alpha \)HER2-huEndo, or \( \alpha \)HER2-huEndo-P125A did not bind to parental EMT6 cells (Fig. 1C-III).

To investigate structural integrity of the endostatin domain, the human endostatin domain of fusion proteins bound to SK-BR-3 and EMT6-HER2 was detected with biotinylated antihuman endostatin and stained with streptavidin-PE conjugate. \( \alpha \)HER2-huEndo and \( \alpha \)HER2-huEndo-P125A were both recognized following binding to SK-BR-3 and EMT6-HER2 by the antihuman endostatin detection antibody (Fig. 1C-V and VI), whereas \( \alpha \)HER2 IgG3 and the isotype control antibodies were not detected.

To determine whether \( \alpha \)HER2-huEndo fusion proteins could also bind to endothelial cells, HUVECs were treated with \( \alpha \)HER2 IgG3, huEndo, huEndo-P125A, or \( \alpha \)HER2-huEndo fusion proteins, and binding detected either with antihuman IgG-FITC, or with biotinylated antihuman endostatin antibody and stained with streptavidin-PE conjugate. Binding of huEndo, huEndo-P125A, and \( \alpha \)HER2-huEndo fusion proteins to HUVECs was readily detected by antihuman endostatin (Fig. 1C-VII), whereas bound \( \alpha \)HER2-huEndo fusion proteins were also readily detected by antihuman IgG-FITC (Fig. 1C-IV). Binding of the isotype control, or of \( \alpha \)HER2 IgG3 was not detected using either reagent. Therefore, \( \alpha \)HER2-huEndo and \( \alpha \)HER2-huEndo-P125A bound to HER2 and the fused endostatin domain(s) bound to endothelial cells.

Inhibition of endothelial tube formation and endothelial cell proliferation

We tested the effects of the \( \alpha \)HER2-huEndo fusion proteins in an \textit{in vitro} angiogenesis assay in which human endothelial cells are plated on Matrigel, and spontaneously aggregate and assemble into multicellular capillary-like tubular structures in response to vascular stimuli (e.g., bFGF, VEGF, fetal bovine serum; refs. 28 and 29). Neither parental antibodies (\( \alpha \)HER2 IgG3 or trastuzumab) nor human endostatin (wild type or P125A mutant type) alone showed appreciable inhibition of tube formation at concentrations tested. In contrast, \( \alpha \)HER2-huEndo fusion proteins, \( \alpha \)HER2-huEndo (Fig. 2E and F), and \( \alpha \)HER2-huEndo-P125A (Fig. 2H and I) strongly inhibited assembly into tubular structures, compared to \( \alpha \)HER2 IgG3 (Fig. 2B), trastuzumab (Fig. 2C), huEndo (Fig. 2D), or huEndo-P125A (Fig. 2G). HUVECs treated with \( \alpha \)HER2-huEndo fusion proteins remained dispersed and exhibited a scattered morphology in dose-dependent fashion (Fig. 2). Inhibition of tube assembly seen with \( \alpha \)HER2-huEndo-P125A (Fig. 2H and I) was significantly greater than that seen for \( \alpha \)HER2-huEndo (Fig. 2E and F) at comparable concentrations, and treatment of HUVEC at 45 nmol/L resulted in complete disruption of tube formation (Fig. 2H and I).

Inhibition of tube assembly has been previously reported with the NC1 domain of collagen XVIII and oligomeric forms of endostatin (17, 18). The increased \textit{in vitro} antiangiogenic effect of \( \alpha \)HER2-huEndo fusion proteins relative to native endostatin may be due to presentation of endostatin as a dimer as previously reported (17, 18). As morphologic changes were observed within 16 hours, this dispersed phenotype was due to scattering of endothelial cells rather than proliferation (division time for endothelial cells \( \geq 24 \) hours).

We also assessed the effects of \( \alpha \)HER2-huEndo fusion proteins on EC proliferation. HUVECs were exposed to increasing concentrations of the fusion proteins for 72 hours in the absence or presence of either VEGF or bFGF. Both wild-type and mutant antibody–endostatin fusion proteins markedly inhibited EC proliferation induced by
either VEGF or bFGF. HUVEC proliferation was more effectively inhibited at comparable concentrations by αHER2-huEndo-P125A than by αHER2-huEndo (\(P = 0.0085\) in the presence of VEGF, \(P = 0.0034\) in the presence of bFGF) or by endostatin alone (\(P = 0.0003\) in the presence of VEGF or bFGF; data not shown).

Serum clearance and stability of HER2-endo
To characterize the pharmacokinetics of αHER2-huEndo, mice were injected i.v. with αHER2-huEndo, αHER2-huEndo-P125A, huEndo, or huEndo-P125A, and clearance of injected proteins was measured by ELISA for human endostatin. Representative results from mice are shown graphically in Fig. 3, and the pharmacokinetic data for all mice are summarized in Table 1. HuEndo and huEndo-P125A were rapidly removed from the serum compartment in mice (\(T_{1/2}\), 2.0–2.1 hours), whereas the \(T_{1/2}\) of αHER2-huEndo and αHER2-huEndo-P125A fusion proteins (\(T_{1/2}\), 40.7 and 57.5 hours, respectively) were significantly increased compared to that of

Figure 1. A, schematic diagram of anti-HER2 IgG3-human endostatin fusion proteins. Details in text. B, expression of anti-HER2 IgG3-CH3-endostatin fusion proteins. Secreted human endostatin fusion proteins were labeled with \(^{[35}S\)methionine and immunoprecipitated and analyzed under reducing and nonreducing conditions. Anti-HER2 IgG3-CH3-β-murine endostatin fusion (αHER2-mEndo) was used as a control (24). C, binding of anti-HER2 human endostatin fusion proteins to HER2 antigen and HUVECs, and recognition by antihuman endostatin antibody. Human breast cancer cells, SK-BR-3 (I, V), murine mammary tumor cells, engineered to express HER2, EMT6-HER2 (II, VI) and EMT6 (III), or HUVECs (IV, VII) were incubated with αHER2-huEndo (filled with gray), αHER2-huEndo-P125A (red line), αHER2 IgG3 (green line), human endostatin (blue line), human endostatin-P125A (red line filled with pink), or isotype control (anti-dansyl IgG3, black line). The bound fusion proteins were identified with either antihuman IgG-FITC conjugated (I–IV) or with biotinylated antihuman endostatin antibody and secondarily stained with a streptavidin-PE conjugate (V–VII).
human endostatin. There is no significant difference in serum elimination between huEndo ($T_{1/2}$ elimination, 2.0 ± 0.4 hours) and huEndo-P125A ($T_{1/2}$ elimination, 2.1 ± 0.2 hours), or between αHER2-huEndo ($T_{1/2}$ elimination, 57.5 ± 3.9 hours) and αHER2-huEndo-P125A ($T_{1/2}$ elimination, 40.7 ± 1.8 hours). Therefore, human endostatin fused with antibody has a serum half-life of at least 20-fold greater than human endostatin alone.

AUC of the human endostatin fusion proteins was increased by a factor of 56 compared with huEndo or huEndo-P125A (1.01 ± 0.11 μg·h/mL or 0.74 ± 0.05 μg·h/mL, respectively) as a consequence of both a longer half-life of elimination and an increased mean residence time (Table 1). The AUC of αHER2-Endo-P125A (293.15 ± 12.78 μg·h/mL) was slightly increased compared with αHER2-huEndo (201.52 ± 14.83 μg·h/mL; Table 1).

Antitumor efficacy in human breast cancer SK-BR-3 xenografts

SK-BR-3 is a HER2-amplified human breast cancer cell line which grows slowly as a xenograft in SCID mice. Trastuzumab, an anti-HER2 IgG1, is able to inhibit the growth of human breast cancer SK-BR-3 alone or in combination with chemotherapy (30). We assayed for antitumor activity of αHER2-huEndo fusion proteins against human breast cancer SK-BR-3 xenografts in SCID mice. Representative experiments are shown in Figure 4. Equimolar doses of protein were injected every other day for 4 weeks (Fig. 4). Endostatin and αHER2 IgG3 did not significantly inhibit tumor growth relative to the nontreated group (PBS, $P = 0.1504$) by day 29, αHER2 IgG3 inhibited tumor growth relative to the nontreated group (PBS, $P = 0.0045$), whereas treatment with αHER2-huEndo or αHER2-huEndo-P125A resulted in significantly greater inhibition of growth ($P < 0.0001$, respectively; Fig. 4A). Mice treated with αHER2-huEndo-P125A showed significantly reduced tumor growth compared to
Figure 4. A, antitumor efficacy of anti-HER2 IgG3-huEndo fusion proteins. SCID mice (n = 5) were implanted s.c. with 2 × 106 SK-BR-3 on day 0, then i.v. injected with anti-HER2-huEndo fusion proteins (42 μg), antiHER2 IgG3 (34 μg), huEndo (8 μg), or PBS every other day (arrows) starting on day 5. Tumor growth was measured as described earlier. The values represent mean ± 95% CI of tumor volume (mm³) of 5 mice. Experiments were repeated 3 times with similar results. A representative experiment is shown. B, survival of mice per treatment group. antiHER2-huEndo (42 μg), huEndo (8 μg), huEndo-P125A (8 μg), antiHER2 IgG3 (34 μg), trastuzumab (30 μg), or PBS every other day (arrows) starting on day 6. Mice with greater than 2500 mm³ tumor volume were euthanized. The proportion surviving in each mouse group (%) is indicated. N. Sig., not significant. (This is a separate experiment from that shown in A.)

### Table 1. Pharmacokinetic variables for human endostatin fusion proteins in mice

<table>
<thead>
<tr>
<th>Variable^a</th>
<th>antiHER2-huEndo</th>
<th>antiHER2-huEndo-P125A</th>
<th>huEndo^b</th>
<th>huEndo-P125Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1/2}$ (h): distribution</td>
<td>0.463 ± 0.023</td>
<td>0.772 ± 0.049</td>
<td>0.060 ± 0.000</td>
<td>0.066 ± 0.000</td>
</tr>
<tr>
<td>$T_{1/2}$ (h): elimination</td>
<td>57.5 ± 3.9</td>
<td>40.7 ± 1.8</td>
<td>2.0 ± 0.4</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>AUC$_{96}$ (μg h/mL)</td>
<td>126.22 ± 4.58</td>
<td>227.40 ± 4.97</td>
<td>0.62 ± 0.01</td>
<td>0.45 ± 0.00</td>
</tr>
<tr>
<td>AUC$_{96}$ (μg h/mL)</td>
<td>201.52 ± 14.83</td>
<td>293.15 ± 12.78</td>
<td>1.01 ± 0.12</td>
<td>0.74 ± 0.05</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>89.4 ± 6.0</td>
<td>58.9 ± 3.1</td>
<td>2.3 ± 0.5</td>
<td>2.3 ± 0.3</td>
</tr>
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</table>

^aFor the pharmacokinetic variables, the superscript 1 represents the distribution phase and the superscript 2 represents the elimination phase. AUC$_{96}$ and AUC$_{96}$ are the first 96 hours and steady-state AUC, respectively. To calculate pharmacokinetic variables, the concentrations of endostatin in serum were fit to a biexponential model ($\alpha$HER2-huEndo, $\alpha$HER2-huEndo-P125A, huEndo, and huEndo-P125A). Data are mean ± SEM.

^bMeasurements of endostatin were made 2 hours after i.v. injection in the mice.

those treated with antiHER2-huEndo ($P = 0.0161$), human endostatin ($P = 0.0343$), or antiHER2 IgG3 ($P = 0.0253$; Fig. 4A). Similar results were seen in 3 separate experiments (Fig. 4A). Treatment with antiHER2-huEndo-P125A completely eradicated tumors after 30 days (Fig. 4A) and no tumor recurrence was observed until the experiment was terminated at day 40 (data not shown).

In separate experiments, mice treated with antiHER2-huEndo-P125A showed improved survival relative to those treated with huEndo ($P = 0.0005$), huEndo-P125A ($P < 0.0001$), trastuzumab ($P < 0.0001$), or antiHER2 IgG3 ($P < 0.0001$; Fig. 4B). Mice treated with huEndo, huEndo-P125A, trastuzumab, or antiHER2 IgG3 all showed significantly improved survival to the untreated group (PBS: $P < 0.0001$), but there was no significant difference between huEndo and huEndo-P125A ($P = 0.2233$) or between trastuzumab and antiHER2 IgG3 ($P = 0.5995$; Fig. 4B).

**Antitumor efficacy requires presence of the HER2 target**

To investigate whether the ability of antiHER2-huEndo-P125A fusion protein to specifically target HER2 enhanced efficacy, BALB/c mice were simultaneously implanted with EMT6 and EMT6-HER2 tumors on opposite flanks. Mice were then treated with either antiHER2-huEndo-P125A, antiHER2 IgG3, or human endostatin (Fig. 5A). antiHER2-huEndo-P125A inhibited EMT6-HER2 tumor growth more effectively than PBS ($P < 0.001$), endostatin ($P < 0.001$), or antiHER2 IgG3 ($P = 0.0001$), but comparable antitumor activity was not seen against EMT6 ($P > 0.05$; Fig. 5B). antiHER2 IgG3 inhibited EMT6-HER2 tumor growth more effectively than PBS ($P = 0.001$), but not than human endostatin ($P = 0.196$; Fig. 5B). Equimolar administration of antiHER2-huEndo-P125A and antiHER2 IgG3 showed preferential growth inhibition of EMT6-HER2, when compared to parental EMT6 implanted on the contralateral flank ($P < 0.0001$, $P = 0.0004$, respectively; Fig. 5C).
whereas PBS ($P = 0.4014$) and endostatin treatment ($P = 0.1665$) showed no significant difference between EMT6-HER2 and EMT6 tumor growth (Fig. 5C). Therefore, selective targeting of HER2 by the fusion protein was required for maximum efficacy.

**Immunofluorescent staining of vasculature of treated tumors**

To investigate the effects of αHER2-huEndo-P125A fusion protein on tumor angiogenesis, histologic sections of tumors were derived from treated and untreated mice after 4 treatments, and tumor vasculature was visualized and total vessel density was quantified using anti-PECAM fluorescence immunostaining (Fig. 6). Immunofluorescent staining of EMT6-HER2 tumors demonstrated that the antibody-endostatin fusion treated group showed thin, short, and fragmented blood vessels on day 12 (Fig. 6B), compared to more extensively arborizing vasculature in the PBS treated group (Fig. 6A). Treatment with αHER2-huEndo-P125A fusion protein caused a statistically significant decrease in total blood vessel density in tumors (Fig. 6C, $P = 0.0038$). To investigate average blood vessel area, total Vd was divided by vessel number to yield average vessel density. Tumors treated with endostatin fusion showed a significant reduction in average vessel density (Fig. 6D, $P = 0.0009$). Treatment with αHER2-huEndo-P125A resulted in a marked reduction in tumor vasculature, as well as profound changes in vascular architecture.

**Discussion**

Antiangiogenic therapy with endostatin has been shown to block tumor growth with little or no emergence of resistance despite multiple cycles of therapy, in a variety of murine models. In several studies, repeated treatment with endostatin resulted in permanent eradication of
tumors (1, 2, 8). However, phase I/II studies of human endostatin did not show the levels of antitumor activity seen in murine models (9–13). We hypothesized that endostatin performance could be improved if the half-life of endostatin could be extended and if endostatin could be specifically targeted to the tumor, to achieve higher local concentrations. In addition, we hypothesized that endostatin might be more effective if delivered as a dimer as in the context of an antibody fusion protein (17, 18). A prototypic anti-HER2-murine endostatin fusion protein retained antiangiogenic activity, prolonged serum half-life compared to endostatin, targeted HER2 expressing tumors, and inhibited in vivo tumor growth which provided initial validation for this concept (24).

To reduce the possible antigenicity of the murine endostatin fusion domain in preparation for human application, we have now constructed 2 new fusions based on human endostatin and on a mutated form of endostatin with increased antiangiogenic properties. Similar to results obtained with a murine endostatin fusion protein (24), human endostatin fused with antibody had a significantly longer serum half-life than human endostatin alone. Yokoyama and colleagues reported that a mutant version of endostatin in which the proline at 125 is substituted with an alanine, showed greater antiangiogenic activity than native endostatin in vitro (19–21). Consistent with this, the αHER2-huEndo-P125A fusion protein showed greater inhibition of tube formation in vitro than either native endostatin, mutant endostatin-P125A, or wild type αHER2-huEndo fusion.

It has been reported that human or murine endostatin treatment inhibits HUVEC assembly into tubular structures in vitro, with cells remaining dispersed and exhibiting a morphology resembling adherent cells on plastic rather than aggregating into characteristic capillary-like tubes (17, 18). Dimers or trimers of endostatin stimulated the motility of endothelial cells, more efficiently than monomers and endostatin oligomerization was required for the efficient inhibition of tube formation (17, 18). Because of the presence of 2 fused heavy chain-endostatin domains in the assembled H2L2 form (Fig. 1B), αHER2-huEndo fusion proteins may effectively present endostatin as a dimer. Dimerization of the endostatin domains could in theory augment binding to integrins, perlecans, and glypicans, further increasing fusion protein activity in vitro and in vivo (17, 18).

Figure 6. Effect of treatment on tumor vascularity. BALB/c mice (n = 4 per group) were implanted s.c. contralaterally with EMT6 and EMT6-HER2 (1 × 10^6 cells per mouse), followed on day 4 by equimolar injections every other day of αHER2-huEndo-P125A (P125A, 42 μg) or PBS. On day 12, mice were sacrificed after 4 treatments. Histologic sections of tumors from the sacrificed mice were analyzed using immunofluorescent staining for PECAM (green). DAPI (blue) was used for counter-staining of the nuclei. Cryosections (n = 5) per each treatment were stained with rat anti-mouse CD31 and anti-rat IgG-Alexa 488 (green fluorescence). Representative immunofluorescent staining of EMT6-HER2 tumor cryosections from mice treated with PBS (A) and αHER2-huEndo-P125A (B) are presented. Blood vessel area (pixel^2, n = 5) in EMT6-HER2 tumors was quantified using NIH ImageJ by color image to form a binary image to allow measurement of blood vessel density, which is presented as total density of vessels (C). Blood vessel area was then divided by number of blood vessels on the field, which is presented as average vessel density (D). The data are presented as the mean of 5 low power field determinations ± 95% CI.
In vivo treatment of established SK-BR-3 xenografts with the αHER2-huEndo-P125A fusion resulted in greater inhibition of growth than αHER2 IgG3, trastuzumab, human endostatin, human mutant endostatin (huEndo-P125A), or αHER2-huEndo fusion protein treatment. The αHER2-huEndo fusion protein specifically targeted tumors expressing HER2 in syngeneic mice simultaneously implanted with EMT6 and EMT6-HER2. Immunofluorescent staining of EMT6-HER2 tumors treated with the αHER2-huEndo-P125A fusion showed thin, short, and fragmented blood vessels. The data presented earlier strongly suggest that the enhanced antitumor effects of the fusion protein may be due to the targeting of antiangiogenic activity by the anti-HER2 antibody domain.

The mutant αHER2-huEndo-P125A fusion inhibited tube formation of HUVEC in vitro, and tumor growth in vivo more effectively than αHER2-huEndo. Four synthetic peptides corresponding to the sequences 6–49 (I), 50–92 (II), 93–133 (III), and 134–178 (IV) of human endostatin have been examined for their ability to inhibit endothelial cell proliferation, migration, and both in vitro and in vivo angiogenesis (31, 32). Fragment I was found to be antiangiogenic whereas unexpectedly, fragment III exhibited a proangiogenic activity, increased endothelial cell migration and neovascularization, and enhanced the angiogenic response to vascular endothelial growth factor in a corneal pocket assay. The P125A point mutation may lead a conformational change that reduces the proangiogenic properties of fragment III. Human endostatin has an internal Asn-Gly-Arg (NGR) motif at position 126–128 of the N-terminal Asp-rich domain (II). The mutant αHER2-huEndo-P125A endostatin bound to endothelial cells more efficiently than wild-type endostatin and exhibited greater inhibition of both proliferation and migration of endothelial cells (20). Vascular endothelial growth factor and angiopoietin 1 were down-regulated more by P125A endostatin than by native endostatin (20). These results suggested that an antibody fusion based on the mutant P125A endostatin might inhibit tumor growth in vivo more effectively than αHER2-huEndo as indeed proved to be the case in our experiments.

Antigenicity of the fusion proteins is a theoretical problem which may affect activity in vivo in either immunocompetent mice or potentially in man, and reduce efficacy. There are several examples of fusions in approved clinical use in which antigenicity has not been a major problem including anti-TNF-fusions (33, 34), DAB2-IL2 (35), and VEGFR-fusion (VEGFR-Trap in patients with solid tumors and non-Hodgkin’s lymphomas; ref. 36). Therefore, the effects of antigenicity on efficacy cannot be predicted a priori from mouse models.

Linking endostatin to an antibody may significantly enhance the antitumor activity of trastuzumab (24). Because the overall response rates of HER2+ breast cancers to trastuzumab remain relatively low (15%–34%; refs. 36–39), this approach holds promise for increasing both response rate and duration relative to trastuzumab, and may expand the spectrum of antitumor activity of trastuzumab given alone or in combination with other antitumor strategies such as other cytotoxic agents (carboplatin, docetaxel; refs. 40–43), and/or antiangiogenic drugs (e.g., bevacizumab; anti-VEGF antibody, thrombospordin-1; refs. 44–47). Experiments are ongoing in our laboratory to determine whether anti-HER2-endostatin fusions may also be active against trastuzumab resistant cell lines in vivo.

Because endostatin is known to be a powerful and global regulator of angiogenic gene expression, we concentrated initial experiments on endostatin as a candidate fusion. In addition to endostatin, other antiangiogenic domains could also theoretically be incorporated into fusions (e.g., angiostatin, tumstatin, etc). Finally, targeting antiangiogenic proteins using antibody is a versatile approach that could be applied to other tumor targets through substitution with other antibody specificities/variable domains. This approach could in theory therefore be applied to enhance efficacy of antibodies directed to other tumor antigens in settings where parental antibody shows modest efficacy (e.g., cetuximab; refs. 48–50).

Disclosure of Potential Conflicts of Interest

S-U. Shin and J.D. Rosenblatt have filed a US patent application.

Acknowledgments

We thank Morgan and Friends (Dr. Herb Krickstein and Morgan Pressel) and the Kassamali Family and Luminaire for the generous gifts and efforts.

Grant Support

The U.S. Army Medical Research and Material Command under W81XWH-05-1-0351 Grant (DOD Idea Award, BCO44744: S.U. Shin), Bankhead Coley Pre-Spore from the State of Florida (S.U. Shin, J.D. Rosenblatt), the National Cancer Institute CA114340 (S. Ramakrishnan).

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Received August 25, 2010; revised November 18, 2010; accepted February 8, 2011; published OnlineFirst March 10, 2011.

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www.aacajournals.org Mol Cancer Ther; 10(4) April 2011

Published OnlineFirst March 10, 2011; DOI: 10.1158/1535-7163.MCT-10-0804


Molecular Cancer Therapeutics

Targeted Delivery of an Antibody–Mutant Human Endostatin Fusion Protein Results in Enhanced Antitumor Efficacy


Mol Cancer Ther 2011;10:603-614. Published OnlineFirst March 10, 2011.

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